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# Protective Immunity of *Plasmodium berghei* in Mice Induced by Repeated Infection and Chemotherapy

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Although spraying of insecticides have played a major role in malaria eradication, further progress with insecticides faces two obstacles; development of DDT-resistance in mosquitoes, and environmental pollution. The increasing level of pollution by chemicals calls for world-wide restriction on the use of chemical sprays, especially DDT and dieldrin, which have seriously affected the ecological balance. Thus, the need to replace insecticides with other methods of malaria control places increased emphasis on the development of successful vaccination techniques.

The immediate task for malarial immunologists is to define the mechanism of protective immunity so that suitable vaccinations can be established. In this context, it seems essential to establish whether or not premunitive immunity (Corradetti, 1963) is the only sound protective response of vertebrate hosts towards plasmodia. Many authors have shown that host immunization by injection of killed malarial parasites does not bring complete protection against challenge infection (Eaton and Coggeshall, 1939; Targett and Fulton, 1965; Cox, 1966; Brown *et al.*, 1970; Schenkel, 1973). On the other hand, immunity to the same strain of *Plasmodium* has been demonstrated in mice surviving a drug-suppressed or diet-suppressed initial infection, and in mice treated with attenuated parasites (Box and Gingrich, 1958; Briggs *et al.*, 1960; Weiss and De Giusti, 1966; Gilbertson *et al.*, 1970; Senger and Jerusalem, 1971). In all of these experiments, the parasite administered as immunizing agent seems to persist in the host for a long period.

It was previously shown (Waki and Suzuki, 1974) that mice rapidly cleared of *P. berghei* by chemotherapy are invariably killed when challenged with the same strain. The aim of the present work was to examine whether protective immunity can be conferred on mice by repeated infection followed by radical antimalarial chemotherapy. The factors essential to protective immunity are discussed.

## Materials and Methods

*Plasmodium*: The NK65 strain of *P. berghei* (Yoeli, 1965) was used. The strain was generously supplied by the late Professor M. Yoeli in 1969, since when the strain has been maintained by blood transfer in mice with occasional freezing at −70 C.

*Animals*: Female DDY strain white mice (Shizuoka Farm, Japan) were employed throughout the experiment. The animals were 5 weeks old at the start of each experiment.

*Drug doses*: Suzuki (1972) showed that 4 consecutive doses of 20 mg/kg/day of sulfamonomethoxine (liquid form) is needed for radical cure of infected mice. The same doses of the drug were administered subcutaneously in the present experiment.

*Immunization procedure*: Mice were inoculated intraperitoneally with $10^7$ parasitized red blood cells. This inoculum always produces 100% mortality in untreated mice. Drug treatment was started on the 3rd day

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| Group | Immunization | Immunization | Immunization | Immunization | Challenge inoculation |
|---|---|---|---|---|---|
| 1. | V | V | V | V | V |
| 2. | | V | V | V | V |
| 3. | | | V | V | V |
| 4. | | | | V | V |
| | Control t. | Control t. | Control t. | Control t. | |
| 5. | V | V | V | V | V |
| 6. | | | | | V |
| | 0 | 2 | 4 | 6 | 8 weeks |

Fig. 1 Immunization of mice by repeated infection and radical chemotherapy.

Immunization: Inoculation with viable *Plasmodium* ($1\times10^7$ parasitized RBC) followed by 20mg/kg/day sulfamonomethoxine (day 3-6)

Control treatment: Inoculation with non-infected RBC with following chemotherapy

Challenge inoculation: Inoculate mice $1\times10^6$ parasitized RBC i.p.

after inoculation and was continued for 4 successive days. The mice so treated showed no parasitaemia on the 7th day after inoculation.

*Experimental schedule*: The immunization schedule is shown in Fig. 1. Seventy-two mice in 6 test groups were used. The mice in groups 1 to 4 were immunized by parasite inoculation followed by chemotherapy 4 times, 3 times, twice and once, respectively, at 2 week intervals. The final immunizations in each group were performed at the same time, 6 weeks after the start of the experiment. The mice in group 5 were inoculated 4 times with normal mouse red blood cells at 2 week intervals and treated with sulfamonomethoxine in the same way as group 1. Another 12 mice were used as untreated controls. A challenge inoculation of $10^6$ parasitized red blood cells was given to each group of mice 15 days after the final immunizing inoculation.

## Results

The survival of parasites in mice immunized by the present procedure was examined on separate groups of mice. The animals were exsanguinated by cardiopuncture 15 days after the final parasite inoculation. Emulsion of the organs (brain, lungs, heart, spleen, liver, thymus, retroperitoneal lumph nodes, kidneys, pancreas, bone marrow, and adipose tissue) and red blood cells from the animals were administered to clean mice by intraperitoneal inoculation. The recipient mice showed no parasitaemia and survived, showing that the animals subjected to infection followed by chemotherapy were in a completely sterile state.

The effectiveness of multiple immunization in developing protective immunity is shown in Table 1. Eleven of the 12 mice immunized 4 times (Group 1) survived challenge with *Plasmodium*; 9/12 of those immunized 3 times (Group 2) and 4/12 of those immunized twice (Group 3) survived the challenge. Group 4, immunized only once, had no significant resistance and had the same mortality (100%) as the untreated control group. Untreated mice (Group 6) died in 6–19 days.

Table 1 Development of protective immunity against *P. berghei* by repeated parasite inoculation and radical chemotherapy

| Group Treatment | Mortality of mice | Mean survival time of fatal cases (days) |
|---|---|---|
| 1. Immunized 4 times | 1/12 | 22 |
| 2. Immunized 3 times | 3/12 | 19 (16-25) |
| 3. Immunized twice | 8/12 | 18 ( 7-25) |
| 4. Immunized once | 12/12 | 10 ( 6-23) |
| 5. Control for drug administration | 12/12 | 11 ( 6-22) |
| 6. Untreated | 12/12 | 8 ( 6-19) |

Thus, protective immunity is evident in all groups immunized more than once, and increases in efficacy with the number of sterile immunizations. This is demonstrated by the longer survival of those mice which ultimately died in groups 1–3, in addition to the lower mortality. Vaccinated mice showed a peak parasitaemia about 10 days after the challenge inoculation ; parasitaemia cleared rapidly thereafter in those mice which eventually survived. However, the level of peak parasitaemia varied considerably within each group, some individuals having a peak level almost as high as the controls. Such cases had several recrudescences at about monthly intervals, though the ratio of parasitized red blood cells never exceeded 1%.

The durability of acquired immunity was examined in mice which had been immunzed 4 times, 3 groups were challenged with $10^6$ parasitized red blood cells 2, 6, and 12 weeks, respectively, after the final immunizing inoculation. The same numbers of non-immune mice of the same age were used as controls. As shown in Table 2, resistance to challenge

Table 2 Persistence of protective immunity to *P. berghei* after accumulated immunizations

| Group | Time of challenge inoculation* | Mortality of mice |
|---|---|---|
| A | 2 | 1/12 |
| B | 6 | 2/12 |
| C | 12 | 1/12 |
| Controls-A, B, C | — | 12/12 |

* weeks after last immunization

was still high as late as 12 weeks after the last immunization, no difference in effecacy of protection being found in the groups challenged at 2 and 12 weeks. All control animals died 6–20 days after challenge.

## Discussion

Protective immunity was found only after repeated infection, and the degree of protection was improved by accumulating the immunization. This result suggested that repeated vaccinations with viable *Plasmodium* is required for acquisition of protective immunity. According to Senger *et al.* (1971), mice repeatedly inoculated with viable *P. berghei* parasites under continuous chloroquine treatment did not show protection against challenge inoculation. However, they also showed that mice cured by the drug during developing parasitaemia showed resistance to challenge, and further pointed out that mice immunized by doses of viable *P. berghei*, whose multiplication was restricted by PABA deficient diet, showed protective immunity even after a single vaccination. Gilbertson *et al.* (1970) obtained similar results with the mice given PABA free diet and suggested that long exposure to latent infection conferred protective immunity on the animals. Barker (1971) reported that mice which had recovered spontaneously from *P. berghei yoelii* infection were immune to the succeeding challenge. A similar result was observed in *P. chabaudi* infection (Cox, 1970). The present results as well as those mentioned above seem to indicate that certain extent of parasite multiplication would be indispensable for establishing a solid immunity in mice.

As regards sterile immunity, Cox (1966) showed protective immunity in mice infected with *P. vinckei* followed by a single infection of chloroquine phosphate. He insisted that the immunity is that of the sterile type, but the mice showed a recrudescence after disappearance of parasitaemia by radical cure, and this recrudescence was effective in acquiring protective immunity. Weiss *et al.* (1966) showed that mice inoculated with *P. berghei* which had been modified by serial passage through tissue culture were subsequently immune to reinoculation with the parent strain without producing an overt primary infection. They also stated that the immunity obtained was of the sterile type. However, sterility was examined only by observing blood films and by subinoculating emulsion of spleen and liver from test animals. The parasite attenuated

through *in vitro* cultivation could have limited multiplication at a level undetected by ordinary techniques; in fact, 2 to 3 months was required for protection to appear in the mice and small number of parasites could have persisted during this period. It looks that a premunitive immunity is reflected in this experiment.

Suzuki (1972) examined by fluorescent antibody technique the survival of parasites in mice that had been infected and cured in the same way as in the present experiment, and found that, the parasite was absent from the brain, lungs, heart, spleen, liver, thymus, retroperitoneal lymph nodes, kidneys, pancreas, intestine, bone marrow and adipose tissue of the host. Recrudescences were not observed in treated mice. As has been further confirmed in the present preliminary examination (see first paragraph of the results), viable parasites could not be found in any of the emulsified organs or blood from the immunized mice. Therefore, the results presented here seem to demonstrate that repeated parasite multiplication confers true sterile immunity to the host animal even after complete elimination of the organism by chemotherapy. The fact that the sterile immunity, once acquired, persisted for at least 3 months without decline of protective potency, will be encouraging for the development of a practical vaccination.

Corwin *et al.* (1969) showed that ducks inoculated with plasma from ducks, chickens and rats infected with several haemosporidia were protected against challenge with both homologous and heterologous parasites. Serological reaction has shown the presence of soluble malarial antigen in the sera of acutely infected man and animals (Smith, 1972). Such antigens are obtained in the natural course of infection and seem to be released from multiplying parasites. Whereas vaccination employing killed parasites or parasite extracts alone are ineffective for inducing protective immunization (Brown *et al.*, 1970; Schenkel *et al.*, 1973). It may be inferred, therefore, that some special substance produced by the parasite only after extensive multiplication is effective as the antigen in acquiring protective immunity.

Most people in endemic areas will have developed a good level of immunity as compared with previously unexposed individuals. Jeffery (1966) observed the effect of prior infection with human *Plasmodium* on subsequent infection in regard to immune response of the host, clinical symptoms, parasitaemia, transmissibility and chemotherapeutic measures. In homologous reinoculations, the symptoms and parasite density of the patients diminished compared with non-immune persons. The present results suggest that acquisition of resistance in individuals or populations is attributable to repeated malaria experience, even if each infection is eradicated by chemotherpy.

## Summary

The development of protective immunity against *Plasmodium berghei* infection in mice has been shown by repeating infections followed by chemotherapy. After each drug treatment the parasites completely cleared from the blood and organs of the host. The acquired immunity observed in the present study was of the true sterile type, and the degree of the protection was pararelled with the repeated times of immunization. Four groups of mice were immunized once, twice, three times and four times respectively. Mice immunized once did not show any significant resistance, whereas 11 mice out of 12 which were immunized 4 times survived and this group of mice showed maximum protective potency against the challenge inoculation. The protective immunity, once acquired, persisted for more than 12 weeks. The immunity was thought to be conferred to mice by repeated parasite multiplication in the host. It may be inferred that some special substance produced by multiplying parasite is effective as the antigen in acquiring protective immunity.

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## References

1) Barker, L. R. (1971) : Acquired immunity to *Plasmodium berghei yoelii* in mice. Trans. Roy. Soc. Trop. Med., 65, 586–590.
2) Box, E. D., and Gingrich, W. D. (1958) : Acquired immunity to *Plasmodium berghei* in the white mice. J. Infect. Dis., 103, 291–300.
3) Briggs, N. T., Garza, B. L., and Box, E. D. (1960) : Alterations of serum proteins in mice acutely and chronically infected with *Plasmodium berghei*. Exp. Parasit., 10, 21–27.
4) Brown, K. N., Brown, I. N., and Hills, L. (1970) : Immunity to malaria : 1. Protection against *Plasmodium knowlesi* shown by monkeys sensitized with drug-suppressed infections or by dead parasites in Freund's adjuvant. Exp. Parasit., 28, 304–317.
5) Corradetti, A. (1963) : In immunity to protozoa Garnham, P. C. C., Pierce, A. E., and Roitt, Ist. ed. Blackwell, London, 69 pp.
6) Corwin, R. M., and Cox, H. W. (1969) : The immunogenic activities of the nonspecific serum antigens of acute hemosporidian infections. Milit. Med., 134, 1258–1265.
7) Cox, F. E. G. (1970) : Acquired immunity to *Plasmodium chabaudi* in swiss TO mice. Ann. Trop. Med. Parasit., 64, 309–314.
8) Cox, F. E. G. (1966) : Acquired immunity to *Plasmodium vinckei* in mice. Parasitology, 56, 719–732.
9) Eaton, M. D., and Coggeshall, L. T. (1939) : Production in monkeys of complement-fixing antibodies without active immunity by injection of killed *Plasmodium knowlesi*. J. Exp. Med., 70, 141–146.
10) Gilbertson, M. M., Maegraith, B. G., and Fletcher, K. A. (1970) : Resistance to superinfection with *Plasmodium berghei* in mice in which the original infection was suppressed by a milk diet. Ann. Trop. Med. Parasit., 64, 495–512.
11) Jeffery, G. M. (1966) : Epidemiological significance of repeated infections and heterologous strains and species of *Plasmodium*. Bull. Wld. Hlth. Org., 35, 873–882.
12) Schenkel, R. H., Simpson, G. L., and Silverman, P. H. (1973) : Vaccination of Rhesus monkey (*Macaca mulatta*) against *Plasmodium knowlesi* by the use of nonviable antigen. Bull. Wld. Hlth. Org., 48, 597–604.
13) Senger, R. C. A., and Jerusalem, C. R. (1971) : Murine malaria. IV. Disturbed immunological responsiveness during *Plasmodium berghei* infection. Exp. Parasit., 30, 41–53.
14) Smith, A. R., Karr, L. J., Lykind, J. D., and Sistic, M. (1972) : Serum-soluble antigens of malaria : A review. Exp. Parasit., 31, 120–125.
15) Suzuki, M. (1972) : Localization of *Plasmodium berghei* in recrudescing mice. WHO-MAL. 72. 778 (unpublished working document).
16) Targett, G. A. T., and Fulton, J. D. (1965) : Immunization of Rhesus monkey against *Plasmodium knowlesi* malaria. Exp. Parasit., 17, 180–193.
17) Waki, S., and Suzuki, M. (1974) : Development and decline of antiplasmodial indirect fluorescent antibodies in mice infected with *Plasmodium berghei* (NK65) and treated with drugs. Bull. Wld. Hlth. Org., 50, 521–526.
18) Weiss, M. L., and De Giusti, D. L. (1966) : Active immunization against *Plasmodium berghei* malaria in mice. Am. J. Trop. Med. Hyg., 15, 472–482.
19) Yoeli, M. (1965) : Studies on *Plasmodium berghei* in nature and under experimental conditions. Trans. Roy. Soc. Trop. Med. Hyg., 59, 255–271.

# *Plasmodium berghei* の感染と治療の反復によるマウスの防禦免疫

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1．マウス（DDY）にマラリア原虫（*P. berghei* NK65）$1\times10^7$ 個を接種し，接種後3日目より連続4日間 sulfamonomethoxine 20 mg/kg/day 皮下注射することにより完全治療を行なつた．この操作を2週毎にくり返し行なうことにより，その後のマラリア原虫攻撃接種に対して防禦免疫が成立した．

2．免疫操作後のマウスの血液及び全臓器乳液を正常マウスに接種することにより，上記免疫マウス体内に原虫の存在しないことが確認された．

3．原虫攻撃（$1\times10^6$）に対する免疫マウスの防禦力の強さは免疫の回数に依存し，免疫操作を4回くり返したマウスの生存率が11/12であつたのに対し，3回のくり返しによつては9/12，2回では4/12，1回及び対照では0/12の生存率がそれぞれ示された．

4．この防禦免疫は免疫操作後12週間経たのちまで全く低下が認められなかつた．

5．従来，マラリアに関しての防禦免疫は，宿主体内に原虫が生存することによつて成立するといわれてきた．（Premunitive immunity）以上の研究結果は宿主体内より原虫を完全に駆逐しきつた状態においても，防禦免疫が成立しうることを明示した．（Sterile protective immunity）